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PROGRESS REPORT

RESEARCH ON PROCEDURES FOR THE
LOW- TEMPERATURE PRESERVATION OF BLOOD

XII

PROCEDURES FOR THE DETERMINATION OF
IN VIVO SURVIVAL OF PRESERVED RED CELLS IN HUMANS

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PROGRESS REPORT ON CONTRACT NONR-3003(00) XII

Procedures for the Determination of In Vivo Survival of Preserved Red Cells in Humans

I. OBJECTIVES AND SCOPE

In previous progress reports (ONR II, ONR IX), we described the procedure for determining in vivo red cell survival in animals and reported a study of the variables. Precision and accuracy of the chromium-51, chromium-51 method were also discussed. A Linde internal report (Technical Memorandum B-316, Dwyer, R. F., "A Chromium-51 Double Tagging Procedure for the Determination of in Vivo Survival of Red Blood Cells", June 8, 1961) has also been issued which extensively treats the subject for animal application. Several parts of this Technical Memorandum have been directly incorporated into the present report.

The objective of this report is only to supply a set of detailed procedures to clinical investigators for the evaluation of survival of preserved blood in humans. There has been no attempt to present any data. If the reader wishes to more extensively learn of the work that lead to the procedures, he must refer to the abovementioned reports.

II. SUMMARY

Many of the existing procedures for the determination of in vivo survival of red blood cells are evaluated and the potential errors of some of these procedures when applied to frozen - thawed blood are discussed.

Three procedures for the in vivo determination of red cell survival in humans are discussed in detail. The first two employ phosphorus-32 and chromium-51. The first was developed by Dr. Clement Finch and co-workers and the second is only a Linde modification of this procedure to make it more applicable to frozen - thawed blood. The third procedure employs a chromium-51, chromium-51 double tagging and was developed in this Laboratory specifically for this project.

Finally, all equipment necessary for any of the three procedures is listed.

III. INTRODUCTION

Several methods are available for determining post-transfusion survival of red cells. In many cases the cells are marked by radioisotopic means before transfusion, and the rate of disappearance from the circulation followed after transfusion. The marking is accomplished so that the transfused red cells can be identified after mixing with the recipient's red cells. Two radioisotopes, phosphorus-32 and chromium-51, can be incorporated into the red cells in vitro and are, therefore, useful for measuring survival of preserved cells (1-9).

As applied to normal red cells, chromium-51 tagging and survival measurements are simple and reliable. Blood is equilibrated with chromium-51 (chromate) and an aliquot of the labeled sample is injected intravenously. Time is allowed for mixing and a blood sample is drawn. Red cell radioactivity in the sample is assayed and taken as the "baseline" or 100% survival figure. At any time "x" another blood sample is drawn and the red cell radioactivity assayed. The specific activity in red cells at time "x" divided by the specific activity in red cells of the early sample times 100 gives the apparent percent survival at time "x".

Red cell circulating volume is determined by a modification in the assaying procedure. By making the additional measurement of the specific activity in the red cells injected and multiplying this by the volume of the red cells injected, the total amount of red cell chromium-51 injected is determined. This is then equal to the specific activity of the red cells sampled after injection (measured) times the circulating red cell volume.

The determination of red cell survival and circulating red cell volume by this method is only as reliable as the measurement of the initial or 100% level of red cell activity after injection. There are at least two factors affecting this 100% level determination. The first is the time necessary to insure proper mixing of the injected sample and the second is the loss of injected red cells. Mixing time varies from individual to individual and in humans may require up to 10 minutes (9). From the standpoint of red cell removal from the circulation, a sample at zero time would be ideal. Strumia (10) recommends taking multiple samples from immediately after injection to about 20 minutes to determine the maximum level of radioactivity.

Measurements of circulating red cell radioactivity following a single injection of chromium-51 tagged cells reflect the composite of a mixing curve and a loss curve. The rate of loss of red cells immediately after injection is unknown and especially in the case of frozen-thawed or otherwise preserved cells may be rapid and marked. Survival values based on a single injection of tagged preserved cells are subject to an uncertainty. This uncertainty results from the fact that red cells may be disappearing from the circulation

during the time mixing is occurring. The experimentally measured maximum early post-injection (100%) level of circulating red cell radioactivity can be significantly lower than it would be if all injected cells remained intact during mixing. Percentage survivals should tend to be maximal values and have inherent in them the assumption that red cell removal from the circulation in the minutes immediately following transfusion is negligible. The opposite is very likely to be true, especially in the case of frozen-thawed blood. The loss curve is likely to be exponential with the highest rate of loss immediately after injection.

In seeking to overcome this limitation various investigators have often calculated the initial or 100% level of activity from independent determination of red cell volume using phosphorus-32 or dye-hematocrit methods (3, 4, 8, 11).

Donohue and Finch (11, 12) employed a survival determination procedure based on the simultaneous injection of normal red cells tagged with phosphorus-32 and preserved cells tagged with chromium-51. Any early loss of the preserved cells would be detected as a change in isotope ratios. Essentially the phosphorus-32 establishes the baseline (100%) survival and the chromium-51 level is compared to it.

A procedure has been developed in this laboratory in which the baseline (100%) survival is established by injecting normal red cells tagged with chromium-51. The survival of the "test" cell is then determined by tagging at a higher level with chromium-51 and performing a second injection.

Both the phosphorus-32, chromium-51 procedure and the chromium-51, chromium-51 procedure have advantages peculiar to themselves. However, for testing red cells in which there is a possibility of significant early in vivo loss, both procedures have definite advantages over other published methods. For this reason, both procedures are detailed in this report and the reader can decide for himself which one he would prefer to employ in his own program.

Of the two radioisotopes, chromium-51 offers several distinct advantages over phosphorus-32. Phosphate labeled cells must be washed and re-suspended in unlabeled medium before injection. Samples must be refrigerated until use and preferably used within a short time of preparation (12). Rapid elution of the isotope occurs in the circulation and multiple samples must be taken to allow correction for elution, or an average elution curve must be established. In contrast, the chromium-51 as chromate enters the red cell rapidly and with high efficiency, and is then bound very tightly with an elution rate of only about 1% per day (11). If the red cell is destroyed or otherwise lost from the circulation, the freed chromium-51 is incapable of retagging

and is rapidly lost from the circulation. This combination of a high efficiency tag, inability to retag, and rapid loss of unbound chromium-51 from the circulation also makes it possible to administer cells in vivo without resuspension in an untagged medium. Also the body burden from chromium-51 is 800 μ c while the body burden for phosphorus-32 is only 30 μ c (13). This makes chromium a much more attractive radioisotope to handle in the laboratory, and also in human infusions.

The Finch procedure in theory is excellent, but in practice is subject to the outlined difficulties with phosphorus-32. In addition, analytical problems arise in measuring, to the same precision, two chemically different isotopes emitting different types of radiation in the presence of each other. Also it has not been proven that phosphorus-32 and chromium-51 isotopes are biologically equivalent under all conditions. To employ a phosphorus-32 tag, the cells must be washed and resuspended before injection. Because of rapid phosphorus-32 elution in vivo, whole blood samples are preferably counted. To make chromium-51 counts comparable, when the two isotopes are used in a simultaneous procedure, whole blood is also counted for chromium-51. Thus, the experimentally treated cells (chromium-51) must be washed before use to avoid extraneous extra-cellular activity. Potentially, this washing of the test specimen could remove the "weaker" cells before testing or so alter viable cells so that they would be immediately removed from the circulation on infusion. The removal of potentially non-viable cells before testing would yield erroneously high survival values while the alteration of normally viable cells would yield low survival values. It would seem preferable to handle the blood as little as possible in the test procedure.

However, the phosphorus-32, chromium-51 procedure also has several advantages over the chromium-51, chromium-51 procedure. Since two different isotopes are employed, the baseline sample (phosphorus-32) and test sample (chromium-51) can be mixed for infusion and still be differentiated. The isotope ratio is established and its shift is noted in the circulating isotope ratio at an appropriate time after infusion. This shift is indicative of the red cell survival. In the chromium-51, chromium-51 procedure, the baseline sample is first infused. Samples are taken from the circulation at the appropriate time to establish the circulating chromium-51 level. Then the test sample is infused and, again at an appropriate time, the circulating chromium-51 level is established. Since the one injection is to be compared to the other on an absolute basis, the infusion volumes must be known quite accurately. This is difficult if survival is to be determined in large transfusions. Since the chromium-51, chromium-51 procedure requires two infusions, it involves more discomfort to the recipient. The two infusions - two sampling system is also more time consuming in the hospital. Whereas the phosphorus-32, chromium-51 procedure requires considerable time for washing and resuspending in the laboratory, the two infusion chromium-51 chromium-51 requires more time from the professional staff.

IV. **PHOSPHORUS-32, CHROMIUM-51 DOUBLE TAGGING PROCEDURE FOR
MEASUREMENT OF POST-TRANSFUSION SURVIVAL OF STORED BLOOD**

The theory of the phosphorus-32, chromium-51 determination of red cell survival is very simple. A sample of unpreserved blood (that is, blood which is collected, "tagged" and transfused without any freeze-thaw treatment, etc.) is labeled with phosphorus-32. Another sample of blood is treated in whatever manner is to be evaluated (such as freeze-thaw) and then labeled with chromium-51. The two samples are then combined, the phosphorus-32 and chromium-51 assayed, and a portion of the combined sample transfused. Samples are subsequently taken from the recipient and the phosphorus-32 and chromium-51 assayed. The phosphorus-32 labeled red cells should be diluted by the recipient's circulating red cells and some may be lost from handling damage. The chromium-51 labeled red cells should also be diluted by the recipient's circulating red cell volume and again some will be lost from damage in handling. The dilution of each isotope should be equivalent. Thus, any difference in the "apparent" dilution is an indication of loss of red cells. This difference is referred to as the red cell survival. The phosphorus-32 label is the baseline and the chromium-51 label is the test specimen.

The procedure has been developed by Dr. Clement A. Finch and co-workers and is described in the literature (12). A modified procedure has been reported by Dr. Finch in a Contract Progress Report (14). The following is a direct copy from this Progress Report as supplied to us by Dr. Joseph F. Saunders, Office of Naval Research, Washington, D. C. In fairness to the authors, nothing has been altered in their presentation of the procedure. However, in our application of this procedure, several modifications have been made, some of which are rather radical and some are minor. Therefore, a second procedure is outlined which includes the modifications, and then finally, a comparison section has been included to aid the reader.



A. Phosphorus-32, Chromium-51 Procedure for Measurement of Red Cell Survival (3)

BLOOD VOLUME - CELL SURVIVAL (1)
(for post-transfusion survival studies)

1. Chromium-51

The specific activity of chromium-51 should be such that the amount of chromium per ml red blood cells is no more than $1\frac{1}{4}$ g/ml rbc. The amount of chromium-51 incubated per recipient is $60\mu\text{c}$. (This will represent an injection of about $40\mu\text{c}/\text{recipient}$.) The sterile sodium chromate (which may be diluted with a convenient amount of sterile saline) is added to a sterile 30 ml tube (or universal donor bottle) which will contain the packed red cells from about 20 ml of blood in ACD from which the plasma has been removed after centrifugation.

Let chromium-51 rbc mixture stand for 1 hour at room temperature mixing several times. Saline (12 cc) is added, blood mixed, and spun down 6 minutes at 3500 rpm in the Servall angle centrifuge. Saline is removed using sterile technique. Repeat 1x. Store in ice until ready.

2. Modified Phosphorus-32 Method of Labeling (2)

About 30 ml of blood (for 14 recipients) is drawn into 0.6 ml heparin*, and 15 ml is transferred into each of 2 screw-capped, sterile containers and centrifuged 6 minutes at 3500 rpm in a Servall angle centrifuge. About 5 to 6 ml of the red cells are aspirated from the bottom of each container (so as to avoid the buffy coat) and transferred to 2 new sterile containers. The cells

(1) Modified from Donohue, D. M. et al., Brit. J. Haemat. 1:249-263 (1955).

(2) Adopted from Mollison, P. L., Robinson, M. A. and Hunter, D. A., Improved method of labeling red cells with radioactive phosphorus. Lancet 1:766-789 (1958)

(3) Appendix I from Erythrocyte Preservation and Metabolism, E. R. Simon, M.D. and Clement A. Finch, M.D., A.D. 255 166, Contract No. DA-49-077-MD-508, Progress Report April 1, 1961

*Heparin - 100 unit/cc

are washed once in 3 volumes of citrate-phosphate-dextrose solution.* Sterile phosphorus-32 in citrate-phosphate-dextrose carrier is added in an amount of 6 to 7 μ c per recipient (about 0.2 cc phosphorus-32 solution per 1.0 cc packed cells). After mixing, the tubes are placed in a water bath at 37°C for 30 minutes. The cells are then washed 3 times with 3 volumes of sterile ice-cold isotonic saline. The last wash is pooled and diluted to 200 cc and an aliquot counted for phosphorus-32. The packed, labeled cells are pooled, their volume is noted and a 0.2 cc aliquot is diluted to 200 cc and an aliquot counted for phosphorus-32. The percent of phosphorus-32 remaining in the supernatant is calculated. The remaining cells are stored in ice until ready.

3. Mixing of Isotopes

Prior to transfusion, the tube containing phosphorus-32 cells is mixed well. Approximately 1 cc of phosphorus-32 packed cells per recipient is then added to the tubes containing the chromium-51 tagged cells. The tubes are then mixed well by rotation and returned to the ice bucket until transfused.

4. Transfusion Procedure

Each tube of blood represents one bag or bottle to be tested (plus one tube of fresh blood drawn that day in ACD as a control and treated in same manner as ACD stored blood). Two recipients are studied per tube, 40% of the amount going to each recipient, leaving 2 cc in the tube for a standard. Usually this means that each recipient will receive 4 to 5 cc of the red blood cell suspension. A tube is removed from the ice, the cells are well mixed and then enough blood is drawn into a sterile syringe for one recipient. The tube is returned to the ice until the next recipient is started. A pre-injection sample is drawn from each recipient (ca. 10 cc), the syringe disconnected, the syringe containing labeled blood attached, and the red cells are injected. (One must work quickly from the time that the blood is removed from the ice until it is injected into the recipient.) Ten minutes after the injection, 30 cc of blood are withdrawn from each recipient and divided into 2 bottles -- one for phosphorus-32, one for chromium-51. About 10 cc of blood are removed on days 1, 2, and 3. All samples are collected in oxalate bottles.

*Citrate-phosphate-dextrose solution is: 3.0 g. trisodium citrate, 0.015 g. sodium di-hydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2 \text{ H}_2\text{O}$), 0.2 g. glucose, water to 100 cc

5. Preparation of Standards and Samples to be Counted

For phosphorus-32 counting, an 11 to 12 cc aliquot of the 10 minute sample, hemolyzed by Saponin,* is counted using a liquid beta ray counter tube (Ekco Electronics type M6H).

For chromium-51 counting, a well-type scintillation counter is used. One pre-injection and duplicate 10-minute samples each of 4 cc are pipetted into 1 dram vials to which a little Saponin has been added for lysis. For the 1, 2, 3-day samples only chromium-51 counts are done on 4-cc samples, treated as above.

Standards

Standards are prepared by diluting 1 to 2 cc of a thoroughly mixed aliquot of the injected cells with distilled water containing carrier phosphate in a 500-cc volumetric flask previously flushed with carrier phosphate to prevent adherence of the phosphorus-32 to the glass walls of the flask.

For phosphorus-32 counting an 11 to 12 cc aliquot is used in the liquid beta counter tube. For chromium-51, duplicate 4-cc samples are put into 1-dram vials for counting with the other samples.

6. Counting

All counting should be performed on the same day to avoid correction for radioactive decay.

Phosphorus-32 counted in liquid tube at least 2x/sample at a predetermined count of 5,000 to 10,000.

Chromium-51 counted on scintillation counter at least 2x/sample at a predetermined count of 2,000.

7. Calculations

(a) All radioactive data, for blood samples as well as standards, are expressed as cpm per sample counted.

*Saponin - Eastman Kodak (Practical) P4704. This was found superior to purified saponin.

(b) Correction for cross-counting is required for the standards prepared from an aliquot of the infused cells and from the 10-minute samples and is performed as follows:

A solution of phosphorus-32 is made up to contain approximately 10,000 cpm as measured in the liquid beta counter. A solution of chromium-51 is made up to contain approximately 5,000 to 10,000 cpm as measured in the well scintillation counter. Approximately 11 to 12 cc of each solution is counted separately in the liquid beta counter. Four milliliters of each solution is counted separately in the well counter. The example below illustrates the calculation for cross-counting.

* Liquid counter results:----- Phosphorus-32 = 12,612 cpm
Chromium-51 = 8 cpm

Well counter results: ----- Phosphorus-32 = 408 cpm
Chromium-51 = 4,836 cpm

Cross-counting of phosphorus-32 in well counter:

$$\frac{12,612}{408} = \frac{1,000}{x}; x = 32 \text{ cpm} = 3.2\%$$

Therefore, for every 1000 phosphorus-32 counts in the liquid counter, 32 counts appear in the well counter.

Cross-counting of chromium-51 in liquid counter:

$$\frac{4,836}{8} = \frac{1,000}{x}; x = 1.6 \text{ cpm} = 0.16\%$$

The chromium-51 count of a sample containing both isotopes is first corrected for phosphorus-32 cross-counting (the greater of the 2) as follows:

Percent (as fraction) cross-counting of phosphorus-32 x phosphorus-32 cpm in sample = A

Chromium-51 cpm in sample - A = chromium-51 cpm corrected for phosphorus-32.

Then the phosphorus-32 count of the sample containing both isotopes is corrected for chromium-51 cross-counting as follows:

Percent (as fraction) cross-counting of chromium-51 x chromium-51 cpm in sample corrected for phosphorus-32 = B.

Phosphorus-32 cpm in sample - B = phosphorus-32 cpm corrected for chromium-51

If the degree of cross-counting of chromium-51 in the liquid counter is of the magnitude given in the example (0.16%) cross-counting due to chromium-51 may be neglected and only the correction due to phosphorus-32 cross-counting is necessary.

(c) In order to obtain the phosphorus-32 cpm per sample recipient's blood at 0 time, the 10-minute value is multiplied by 102% to correct for the loss of phosphorus-32 during the first 10-minutes.

(d) Theoretical 100% survival (chromium-51 cpm per sample recipient's blood) =

$$\frac{\text{Cr}^{51} \text{ cpm per sample standard}^*}{\text{P}^{32} \text{ cpm per sample standard} \times 0.98} \times \text{P}^{32} \text{ cpm per sample recipient's blood at 0 time}$$

* Corrected for phosphorus-32 cross-counting

** The counting rate of phosphorus-32 in blood is 0.98 times the counting rate of phosphorus-32 in the water standard with the equipment being used.

(e) Actual survival at time t (per count) =

$$\frac{\text{Cr}^{51} \text{ cpm per sample recipient's blood at time t} \times 100}{\text{Theoretical 100% Cr}^{51} \text{ survival} (\text{Cr}^{51} \text{ cpm per sample recipient's blood})}$$

The values for each day are plotted on semilogarithmic paper and a straight line extrapolation made to 0 time. The percent survival thus obtained is the value designated to represent the post-transfusion survival.

**B. Linde Modified Phosphorus-32, Chromium-51 Procedure
for Measurement of Red Cell Survival**

1. Chromium-51

The specific activity of chromium-51 should be such that the amount of chromium is no more than $1\mu\text{g}$ per cc rbc. The amount of chromium-51 incubated per recipient is such that about $90\mu\text{c}$ will be infused. The sterile sodium chromate (which may be diluted with a convenient amount of sterile saline) is added to the preserved blood in a sterile container.

Let chromium-51 red blood cell mixture stand for 30 minutes at room temperature mixing several times.

2. Phosphorus-32 Method of Labeling

About 30 cc of whole blood is collected (from the future recipient) in an Abbott bottle containing 10 cc of ACD. The blood is spun for thirty minutes in a refrigerated centrifuge and the supernatent and buffy coat aspirated off the packed red cells. The cells are washed once in 3 volumes of citrate-phosphate-dextrose solution.* Approximately $60\mu\text{c}$ of phosphorus-32 is added to the packed cells after removal of the wash solution and is equilibrated for 30 minutes at 37°C . (This will result in an administered dose of $5-10\mu\text{c}$ phosphorus-32.) The cells are then washed 3 times with 30 cc volumes of sterile ice-cold isotonic saline. If time does not permit transfusion the same day, the resuspended cells can be held overnight in a refrigerator (4°C) and washed once more before use.

3. Mixing of Isotopes

Prior to transfusion the phosphorus-32 tagged cells are resuspended in isotonic saline and 10 cc transfused to the bottle containing the chromium-51 tagged cells. After sufficient mixing, the phosphorus-32, chromium-51 mixture is returned to the ice until transfused.

4. Transfusion Procedure

Small Volume

The bottle is removed from the ice and 20 cc of blood is drawn into a sterile syringe and immediately infused into the recipient via a convenient vein in the antecubital region. (The recipient is the donor for the phosphorus-32 tagged blood. In our work, he is also the donor for the chromium-51 tagged "test" blood.) The bottle is returned to the ice. Ten

*Same as original procedure reagent.

minutes after the injection 5 cc of blood is withdrawn from the recipient and transfused to a tube containing dry oxalate anticoagulant. It is iced until further treatment. Samples are also taken at 24, 48, and 72 hours after infusion.

Large Volume

If it is necessary to determine the red cell survival on 1/2 pint or one pint volumes, 5-7 cc of the phosphorus-32 tagged blood is added to the chromium-51 tagged test blood and thoroughly mixed. A representative 15 cc sample is then removed for radioassay, and iced until used. The container and transfusion kit are weighed, a normal transfusion procedure followed, and the empty container and transfusion kit reweighed. The density of the transfused blood should be determined on the removed sample at the same time that the radioassay sample is prepared so that the transfused volume can be calculated.

5. Preparation of Standards and Samples to be Counted

For phosphorus-32 counting a well-type scintillation system (anthracene crystal) is employed. The scaler is a Model 131A, Baird Associates, Cambridge, Massachusetts.

For chromium-51 counting a well-type scintillation system (sodium iodide crystal) is employed. The scaler is a Nuclear Chicago Automatic Well Counter.

Standards (preinjection assay samples) are prepared by sampling 3 cc from the phosphorus-32, chromium-51 mixture, centrifuging it for 20 minutes, removing the supernatent, quantitatively diluting the cells to 250 cc and transferring 1 cc of the diluted material to a counting tube for radioassay. All glassware has been rinsed with a phosphate carrier and the volumetric flask contains a phosphate carrier solution. If a 1/2 pint or one pint transfusion was conducted, the dilution should be to 50 cc rather than 250.

The post-injection radioassay samples are prepared by centrifuging the samples taken from the recipient after transfusion and transferring 1 cc of packed cells to a counting tube*. (These tubes have a wall thickness of only 25 mg/cm² to allow the beta emission from phosphorus-32 to travel to the scintillator.)

The preinjection samples and 10 minutes post-injection samples are assayed for both phosphorus-32 and chromium-51. The 24, 48, and 72 hour post-injection samples are only assayed for chromium-51.

*See chromium-51, chromium-51 procedure for sampling 1 cc packed cells.

6. Counting

All counting should be performed on the same day to avoid correction for radioactive decay.

Phosphorus-32 counted for 3 minutes.

Chromium-51 counted for 3 minutes or 10,000 counts, whichever is shorter.

7. Calculations

(a) All radioactive data, for blood samples as well as standards, are expressed as cpm per sample counted.

(b) Normally no correction is necessary for cross-counting.

Phosphorus-32 interference on chromium-51 counter = 0.76%

Chromium-51 interference on phosphorus-32 counter = 0.45%

(c) In order to obtain the phosphorus-32 cpm per sample of the recipient's blood at 0 time, the 10-minute value is multiplied by 1.02 to correct for the loss of phosphorus-32 during the first 10 minutes.*

(d) Theoretical 100% survival (chromium-51 cpm per sample of recipient's blood) =

$$\frac{\text{chromium-51 cpm per sample standard}}{\text{phosphorus-32 cpm per sample standard}} \times \frac{\text{phosphorus-32 cpm per sample recipient's blood at 0 time}}{\text{sample recipient's blood at 0 time}}$$

(e) Actual survival at time t (%) =

$$\frac{\text{chromium-51 cpm per sample recipient's blood at time } t}{\text{theoretical 100% Cr}^{51} \text{ survival}} \times 100$$

*This elution rate should be checked initially by each investigator since variations in handling may affect the stability of the phosphorus-32 tag. Samples for phosphorus-32 counting should be taken from the recipient at intervals of about 2, 5, 10, 20 minutes and the phosphorus-32 assay plotted against the sample time on semilogarithmic paper. Once the elution rate has been established, it can be used in "step c" and need no longer be determined.

C. Major Differences Between Original and Modified
Phosphorus-32, Chromium-51 Procedures

1. In the original procedure the "test" cells are washed after tagging with chromium-51. In the modified procedure they are not washed. This washing was excluded in the modified procedure because we wished to test preserved red cells with a minimum of handling after preservation and before transfusion. If the cells were slightly damaged during the preservation process, any washing might conceivably remove the damaged population and the survival data would be erroneously high, or the preserved cells might be more sensitive to saline resuspensions and the additional damage would lower the survival values.

2. In the original procedure, whole blood is radioassayed. In the modified procedure packed cells are radioassayed. Since it is the cell fraction of the whole blood that is being evaluated it seemed more logical to assay the cells. Also, since in the modified procedure, the chromium-51 tagged blood is not washed and resuspended, there is extraneous chromium-51 in the plasma. This would interfere in the assay.

If there is considerable lysis immediately after transfusion, the original procedure would not as readily detect it as the modified procedure. The chromium-51 released from the cell upon lysis would still be in the circulating plasma at 10 minutes and, therefore, would still be in the assay sample if whole blood is assayed. On the other hand, if the phosphorus-32 loss from the baseline cells is excessive, the original procedure would not suffer as badly as the modified procedure for the same reason that most of the phosphorus-32 loss from the cells would still be in the plasma fraction of the sample.

3. In the original procedure the cells are sampled from under the buffy coat before phosphorus-32 tagging. In the modified procedure the plasma and buffy coat are removed together. This does not seem to be a significant change. It was only done to ease the problem of maintaining sterility in the Abbott bottles and to simplify equipment. The same type of aspiration kit can be used for the removal of the buffy coat as is used for removing the wash solutions.

4. In the original procedure a single source of phosphorus-32 tagged blood is used for the baseline determination on as many as 15 recipients transfused in one day. The modified procedure uses each individual recipient's blood for his phosphorus-32 tagged baseline. If the pooled baseline blood used in the original procedure is excessively damaged in any manner, all the survival results from that day will be high, and in studying preserved cells which do not have a 100% survival rate, the investigator may not be aware of the error. By the modified procedure,

an error in a baseline sample would affect only the one result, and would be manifested as an increase in the standard deviation when comparing many transfusions of blood preserved in a single manner. Also there is less risk to the patient in using his own blood for the baseline. Similarly, if the preservation procedure is to be tested on humans not requiring blood it is better to use the recipient's own blood as the test specimen. Autologous transfusions are inherently safer to the patient.

5. The original procedure employs a dipping counter to assay phosphorus-32. The modified procedure employs a well scintillation system. By the modified procedure only a single counting sample is needed for both phosphorus-32 and chromium-51 radioassays since the chromium-51 is also determined by a well scintillation system. A single sample is not only simpler but also more accurate, since the sample volume is not as critical if two samples do not have to be compared. (This is only true of the pre-injection samples and the 10 minute post-injection samples; the 24, 48, 72 hour samples are compared to each other.)

V. CHROMIUM-51, CHROMIUM-51 DOUBLE TAGGING PROCEDURE FOR THE DETERMINATION OF IN VIVO SURVIVAL OF HUMAN ERYTHROCYTES

In an attempt to utilize the better points and to avoid some of the weaknesses of previous work, we have devised a two-injection procedure utilizing only chromium-51. It involves the comparison of the behavior of a tagged sample of untreated blood (baseline) to that of a tagged sample of test blood in a common recipient. Since it is a direct comparison, the only variable studied is that treatment of the test sample, after donation, which is dissimilar to the treatment of the baseline blood.

A sample of blood, usually the recipient's own, is tagged with chromium-51 in the accepted manner and a known volume transfused on the morning in which the test blood is to be evaluated. The red cells are separated from a second aliquot of this sample, and radioassayed to determine the amount of bound chromium-51 transfused. A sample is taken from the circulation after the transfusion, usually at thirty minutes to allow time for in vivo equilibrium. One cc of packed red cells is separated from each sample and radioassayed.

The first injection involves only blood that has received no treatment other than that involved in collection, tagging, and reinjection. It should, therefore, undergo minimum loss in thirty minutes. If the total bound chromium-51 radioactivity is divided by the specific activity of the thirty minute sample, one obtains the dilution factor of the system or a value proportional, if not equal, to the circulating red cell volume.

The test specimen is tagged and injected in a similar manner, except the tag level is three-fold higher. If red cell survival in this specimen is 100%, the activity of the test specimen circulation samples will yield the same dilution factor as the baseline injection. Any increase in this dilution factor is a measure of the extraordinary loss of the red cells undergoing assay. Samples taken at 24 hours or later will indicate any additional loss in the longer time interval.

One of the major points for consideration in this procedure is the fact that all radioassays are performed on packed red cells, not whole blood as is usually the case in other procedures. This has several decided advantages. In assaying an aliquot of all the cells in a sample equivalent to the injection sample, the total red cell bound chromium injected is determined without using a hematocrit. In most other procedures one cc of whole blood is assayed before injection (4, 5, 6) and the red cell activity is then determined by multiplying the total activity by the hematocrit. This introduces the potentially large error in the determination of micro hematocrits. Again in our procedure, one cc amounts of the packed red cells from the circulation samples are assayed rather than one cc of whole blood. This limits the assay to only the red cell bound chromium. Also, by assaying only red cells,

the procedure is insensitive to fluctuations in plasma volumes.

Whenever packed cells are sampled, the trapped plasma must be taken into consideration. It has been reported that as much as 7% of the packed red cell volume may be plasma*. However, this trapped plasma will not seriously affect the survival determination. In the sample to be injected, the specific activity of the plasma is usually only about 1/10 to 1/50 of the specific activity of the packed red cells. In the radioassay of all the packed red cells from the sample representative of the injection sample, as much as 0.2 cc of plasma may be included with the cells. This includes 7% trapped plasma (0.1 cc) and about 0.1 cc of supernatent plasma left over the cells in the separation procedure (see Detailed Procedure). However the 0.2 cc of plasma will only contribute at most 1% additional radioactivity to the cells and is usually consistent from the baseline to the test samples and is therefore canceled. There is also trapped plasma in the packed cells from samples taken from the recipient after infusion. The chromium-51 content of this plasma is negligible when compared with the total chromium-51 in the cells. Therefore the only error is the 7% plasma dilution of the cells referred to earlier. This would cause an error in red cell volume determinations, but is again an error which is canceled in survival determinations because of the comparison of identically handled circulation samples for both the baseline and test parts of the procedure.

Gray and Sterling (2) have applied a very similar procedure to the determination of red cell volumes. They also assay only the packed cell fraction of the whole blood and ignore the trapped plasma in the cells. The accuracy of their method was verified by a second chromium-51 determination of the red cell volume of the recipient after the transfusion or hemorrhage of a known volume of red cells. The results agreed with the predicted change in red cell volume within 3%. The Linde procedure, as outlined, also involves only the determination of successive red cell volumes. The ratio of apparent red cell volumes (utilizing baseline and test sample injections) indicates the fraction of surviving red cells in the test specimen. Therefore the work of Gray and Sterling affords an excellent corroboration of our procedure. Also, Reeve, et al. (15) used chromium-51 tagging to determine successive blood volumes on dogs with equal success.

The test specimen is tagged at a higher level than the baseline specimen for a definite reason. The recipient will retain most of the chromium-51 injected during the baseline measurement and therefore will have a "background" in his circulation. In preliminary discussions on a proposed procedure this appeared to present a problem. However, in our assay method this background is only about 33% of the activity injected into the circulation

*unpublished work in this laboratory

with the test sample and, therefore, can be subtracted without unduly affecting the accuracy of measuring the circulating radioactivity after the test sample. Further, even if 6% of the baseline injection is lost from the circulation in 24 hours (when the next samples are taken) it will only appear as 2% loss to the test sample.

Detailed Procedure

1. The procedure applied for the collection of the blood for the test sample is dependent upon the preservation procedure, but at least 30 cc should be available for a survival determination after the preservation procedures being tested have been completed.

If the preserved or test blood is to be evaluated within 24 hours of collection and the donor is to be the future recipient of the same blood, and if additives are not present in the collection bottle (except ACD anti-coagulant) collect 30 cc of whole blood in excess of that required for the test sample. After collection and mixing, transfer 30 cc of the whole blood to an empty sterile Abbott bottle (or any other 50-150 cc volume siliconized sterile bottle with septum closure) for use as the baseline sample. Store this sample at 4°C until approximately two hours before the test sample is to be evaluated. With this procedure the test and baseline samples are collected in an identical manner.

When the test blood is not to be evaluated within 24 hours of collection or the donor and recipient are two different individuals or if there are special additives in the test collection bottle, collect 30 cc of whole blood directly from the future recipient to the test blood within 24 hours of use. This can be done by gravity collection through a standard Baxter Donor Set to a vented Abbott bottle.

2. Approximately one hour before infusion, remove the baseline blood from refrigeration and add 50 μ c of sodium rachromate and equilibrate at room temperature for 30 minutes. After equilibrium add 50 mg of ascorbic acid solution to reduce excess chromium. Equilibrate 10 minutes.

3. After equilibration, mix the baseline whole blood thoroughly and withdraw exactly 10 cc into a precision syringe for infusion. Using an 18 gauge needle transfuse this 10 cc into the patient via a convenient vein in the antecubital region. Elevate the arm for a moment to insure the rapid circulation of the infused sample. Suitably tag the arm infused as the "in" arm to prevent inadvertent sampling from this arm. It is important that the blood sample be well mixed and that an exactly known volume is infused so that the assay samples will represent a known fraction of the infused radioactivity.

4. Immediately after infusion*, remove two 5.0 cc samples (by precision syringe) from the Abbott bottle and transfer to conical centrifuge tubes. Again it is imperative that in vitro mixing be complete and that a known volume is sampled.

5. Centrifuge these duplicate samples for 20 minutes at approximately 3000 rpm, remove most of the supernatant plasma leaving about 0.1 cc above the cells. Be very careful not to remove any cells. It is important that all samples from a given individual be centrifuged in one centrifuge to avoid variable packing.

6. Transfer the packed cells from each sample to 100 cc volumetric flasks and dilute to volume with water. Transfer 1.0 cc samples from each volumetric flask to the counting tubes. This 1.0 cc can be measured with the same type of tuberculin syringe as will be used on the packed cell samples later in the procedure.

The radioactivity in these samples is 1/100 of the cell-bound chromium present in 5.0 cc of whole blood. Since 10.0 cc of identical whole blood was infused, the total cell-bound chromium infused is 200 times that in the counting tube.

Samples S_D^1 and S_D^2

7. (Refer to Step 3.) Thirty minutes after infusion take approximately 10 cc blood from a vein in the opposite arm. This sample can be taken into a disposable syringe through a 20 gauge needle. Transfer to a sample tube containing dry oxalate anticoagulant.

8. Divide the sample roughly into equal fractions and transfer to conical centrifuge tubes. Centrifuge as in Step 5. With a tuberculin syringe, transfer 1.0 cc of packed cells from each sample to counting tubes.

To aid in the sampling of the packed cells remove all of the supernatant plasma and some of the cells before withdrawing the specimen. Lubricate the syringe plunger with water before attempting to draw packed cells. The syringe is most easily filled by slowly drawing up about 0.9 cc of cells and then, without removing the syringe, slowly expelling them

*As a note of caution, each whole blood sample should be processed immediately for radioassay. If the samples are allowed to stand, the cells may shrink, expand, or hemolyze. Once in the "counting tubes" they can stand any length of time. In our work, one person conducts the infusions while a second processes the samples.

again to the pool of cells. This will remove the air bubble trapped at the base of the plunger. One cc of packed cells can then be drawn into the syringe without entrapment of bubbles. Be sure to make all withdrawals slowly as the high viscosity packed cells tend to degas if suddenly exposed to a reduced atmosphere.

Samples S_R^1 and S_R^2

9. If the test blood was not tagged prior to preservation, it should be tagged concurrently with the previous steps or at this point in the procedure.

The most accurate method of evaluating red cell survival is to transfuse only a small volume, in this case 10.0 cc. In so doing the circulating red cell volume of the recipient is not altered significantly. If a 10.0 cc sample is to be transfused, transfer 40 cc of the test blood to a sterile siliconized Abbott bottle and add 300 μ c of sodium rachromate. (This represents 75 μ c delivered dose to the recipient.) After 30 minutes equilibration at room temperature add 50 mg of ascorbic acid and reequilibrate 10 minutes.

If the red cell survival is to be determined in conjunction with clinical observation of the recipient after the transfusion of one pint of preserved blood, the whole pint should be "tagged". If the total quantity is to be transfused, add 75 μ c of sodium rachromate, (if a fraction of the total quantity is to be transfused add enough chromium-51 to provide for the transfusion of 75 μ c) equilibrate 30 minutes, etc.

10. After equilibration, mix the test sample thoroughly and remove two 5.0 cc samples for assay. Again, as in Step 4, the mixing and volume measurements must be done carefully. Repeat Step 5.

11. Transfer the packed cells from each sample to 250 cc volumetric flasks and dilute to volume with water. Again, transfer 1.0 cc samples from each volumetric flask to the counting tubes.

Samples T_D^1 and T_D^2

12. Transfuse the test sample.

The test sample should be infused within a reasonable time after the baseline injection. Generally, we space the injections no more than four hours apart.

(a) If 10.0 cc is to be transfused, mix the sample well, measure 10.0 cc into a precision syringe and infuse into the same arm as the baseline injection.

(b) If a large volume is to be transfused, weigh the blood, container, and Baxter Transfusion Set on a laboratory double pan balance. Transfuse the entire sample (as far as practical) and reweigh the container and Transfusion Set. The difference is the weight of blood transfused. The density of the blood is determined by weighing the 5.0 cc samples from Step 10; that is, after the blood is drawn into the syringes, weigh the syringes on an analytical balance, deliver the measured volume to the centrifuge tubes and reweigh the syringes. The difference in weight is the weight of 5.0 cc of test blood. Knowing the density, the transfused weight can be converted to volume.

13. Repeat Steps 7 and 8.

Samples T_R^1 and T_R^2

14. After 24 hours repeat Steps 7 and 8 for an evaluation of red cell survival 24 hours after transfusion. If other time intervals are to be studied, repeat as needed.

15. Each sample is radioassayed in the Gamma, Well Scintillation Counter for at least 1 minute or 10,000 "counts", whichever requires the greater time.

Derivation of Equation for Red Cell Survival

The theoretical basis for the calculation of red cell survival involves a material balance; the injected radioactivity is equal to the total circulating radioactivity divided by the survival.

1. Total radioactivity injected as a red cell "tag" in the baseline injection =

$$(S_D) (100) \left(\frac{10}{5}\right)$$

S_D = average, net counts/minute from 1/100 aliquots of diluted cells from 5.0 cc whole blood samples

$$= \frac{(S_D^1 - BKG) + (S_D^2 - BKG)}{2}$$

(Procedure Step 6)

$$\frac{10}{5} \frac{10 \text{ cc infused}}{5 \text{ cc sample}}$$

BKG = Background "count" of the Gamma Counter used in the measurement of radioactivity

2. Total red cell tag in the recipient thirty minutes after baseline injection =

$$(S_R) (V_S)$$

S_R = average, net counts/minute from 1.0 cc packed cells from samples taken from circulation 30 minutes after infusion

$$= \frac{(S_R^1 - BKG) + (S_R^2 - BKG)}{2}$$

(Procedure Step 8)

- V_S = apparent red cell volume after baseline injection

3. Considering a material balance, the total radioactivity injected is equal to the total radioactivity in the recipient corrected for any loss of tagged red cells before samples were taken from the circulation.

$$(S_D)(100)(2) = \frac{(S_R) (V_S)}{\% \text{ Survival of Baseline Cells}} \cdot \frac{100}{100}$$

4. Similarly, total radioactivity injected as a red cell tag in the test specimen =

$$(T_D) 250 \left(\frac{V_X}{5} \right)$$

- T_D = average, net counts/minute from 1/250 aliquots of diluted cells from 5.0 cc samples of whole blood

$$= \frac{(T_D^1 - BKG) + (T_D^2 - BKG)}{2}$$

(Procedure Step 11)

V_X = volume of test blood transfused

5. In assaying the post-infusion samples after the test injection, the "counts" must be corrected for the counts remaining in the circulation from the baseline injection in order to obtain the true response of the injected test cells.

If a large volume transfusion is employed in the test, circulating specific activity in the red cells from the baseline injection (S_R) will be lowered by dilution.

The volume of cells added during the test injection =

$$V_x (Hm \ t \ c) = V_c$$

Hm t c = hematocrit of test injection

. . . Corrected circulating activity from S_D infusion =

$$(S_R) \left(\frac{V_s}{V_c + V_s} \right)$$

If a small volume transfusion is employed $\frac{V_s}{V_c + V_s}$ approaches unity and the correction can be ignored.

Total radioactivity from the test injection still remaining 30 minutes after test injection (as a red cell tag) is as (2)

$$= \left[T_R - (S_R) \left(\frac{V_s}{V_c + V_s} \right) \right] V_T$$

T_R = average, net counts/minute from 1.0 cc packed cells from samples taken from circulation 30 minutes after infusion

$$= \frac{(T_R^1 - BKG) + (T_R^2 - BKG)}{2}$$

(Procedure Step 13)

V_T = apparent red cell volume after test injection

6. As in (3)

$$(T_D) (50) (V_x) = \frac{\left[T_R - (S_R) \left(\frac{V_s}{V_c + V_s} \right) \right] V_T}{\frac{\% \text{ Survival of Test Cells}}{100}}$$

7. If a small volume test transfusions are employed we can assume $V_s = V_T$ within experimental error. If large volume transfusions are employed

$$V_s = V_T - V_c$$

For purposes of this derivation the correction shall be included, but it can be dropped from the final formula if small volume transfusions are employed.

8. From (3)

$$V_S = \frac{(SD)(100)(2)}{S_R} \quad \frac{\% \text{ Survival of Baseline Cells}}{100}$$

9. From (7) and (8)

$$V_T = \frac{(SD)(100)}{S_R} \quad \frac{\% \text{ Survival of Baseline Cells}}{100} + V_C$$

10. Substituting V_T in Equation 6 and rearranging

$$\frac{\left[T_R - (S_R) \left(\frac{V_S}{V_S + V_C} \right) \right] \left[(SD)(100)(2) \frac{\% \text{ Survival of Baseline Cells}}{100} + V_C \right]}{S_R} = \frac{\% \text{ Survival of Test Cells} = (100)}{T_D(50)(V_x)}$$

11. The baseline cells have suffered no damage by test conditions such as storage or freeze-thaw processing. They will exhibit a survival equivalent or superior to the maximum survival of test cells obtainable by any preservation procedure. Therefore, their survival can be acceptably used as a reference level (100%) against which alteration of survival of test cells induced by any preservation technique can be measured.

This procedure of comparison would be valid regardless of losses ascribable to other than actual preservation process conditions since such losses would be expected to apply to both baseline and test cells. However, in our assay, the baseline cells are the recipient's own and have undergone a minimum of handling. They should approximate, as closely as is experimentally attainable, 100% survival on an absolute basis.

$$\therefore \frac{\% \text{ Survival of Baseline Cells}}{100} = 1$$

And equation 10 is reduced to

$$\frac{\% \text{ Survival of Test Cells} = 100}{\left[\frac{T_R - (S_R)}{T_D (50) (V_x)} \left(\frac{V_S}{V_S + V_C} \right) \right] \left[\frac{(S_D) (100) (2)}{S_R} + V_C^* \right]}$$

or in small volume test transfusions

$$\frac{(T_R - S_R) (S_D) (100) (100) (2)}{(50) (T_D) (S_R) (V_x)} \quad \frac{(T_R - S_R) (S_D) (400)}{(T_D) (S_R) (V_x)}$$

The % Survival of test cells at times other than 30 minutes after infusion are calculated by substituting the assay data on samples taken from the circulation at the time in question in place of the assay data of the 30 minute samples.

*In our normal work the formula is not reduced to this simple form. The duplicate assays are not averaged prior to this point and background is not yet subtracted. However, all steps in the calculation are programmed on a Burrows Datatron 205 Computer and the counting data is sent directly from the automatic counter to the computer for calculation.

VI. APPARATUS

Model DS 5-5 Scintillation Well Counter with Model 186 Decade Scaler and
(*Model 1810 Radiation Analyzer)

This can be made automatic by the addition of

Model C 120 Automatic Changer and Model C 111B Printing Timer
Nuclear Chicago Corporation, Des Plaines, Illinois

*Model 131A Scaler with Beta Particle Anthracene Well Counter
Baird Associates, Cambridge, Massachusetts

Model CL International Clinical Centrifuge (one per patient transfused each day)
International Equipment Company, Boston, Massachusetts

Glass Counting Vials R. P. C. Model A
R. P. Cargille Laboratories Inc., 117 Liberty Street, New York, New York

*Plastic Counting Vials - Thin Wall E-52
Tracerlab, Inc., Waltham, Massachusetts

ACD Bottles 100 cc List 6761
Abbott Laboratories, Oak Ridge, Tennessee

Analytical Balance Type DLB, capacity 200 gms, sensitivity 1/20 mg
Wm. Ainsworth and Sons, Inc., 2151 Lawrence St., Denver 5, Colorado

Balance, double pan Model 2-090, capacity 2000 gms, sensitivity 0.1 gms
Fisher Scientific Co., 711 Forbes Avenue, Pittsburgh, Pennsylvania

Centrifuge Tube 15 cc, Conical Bottom Model 5-495R
Fisher Scientific Co.

Tuberculin Syringes, one cc, precision
Becken, Dickinson and Company, Rutherford, New Jersey

B-D Yale, Luer-Loc Syringes, 5 cc and 10 cc
Becken, Dickinson and Company

Disposable Syringes 5 cc, 10 cc
Disposable Hospital Products Inc., South San Francisco, California

Blood Administration Set No. 4517
Abbott Laboratories, North Chicago, Illinois

Blood Collection Set No. 4736
Abbott Laboratories, North Chicago

Plexitron-Plasma Aspirating Set No. R32
Baxter Laboratories, Inc., North Grove, Illinois

Sodium Rachromate List 6716
Abbott Laboratories, Oak Ridge

***Sodium Radio-Phosphate List 6710**
Abbott Laboratories, Oak Ridge

ACD (Anticoagulant)

26.4 gms $C_3H_3(OH)(COONa)_3 \cdot 2 H_2O$
26.8 gms Glucose (Dextrose, Anhydrous)
9.6 gms $C_3H_3(OH)(COOH)_3 \cdot H_2O$

Dilute to 2000 cc with H_2O

Oxalate Solution (an Anticoagulant)

1.68 gms $K_2C_2O_4 \cdot H_2O$
0.5 gms $H_2C_2O_4$
Dilute to 100 cc with H_2O

Ascorbic Acid Solution - 0.1 gms/ml isotonic saline (U.S.P.)
Merck Company, Rahway, New Jersey

***Saponin - Practical - List P4704**
Eastman Kodak Company, Rochester, New York

***Equipment not necessary for chromium-51, chromium-51 Procedure, only**
phosphorus-32, chromium-51 Procedure.

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